

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

933-149PCT

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/424080

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/FI98/00418

18 May 1998

19 May 1997

TITLE OF INVENTION

COMPOSITIONS FOR ENHANCING IMMUNOSUPPRESSANTS PHARMACEUTICAL ACTIVITIES

APPLICANT(S) FOR DO/EO/US

ZAVIALOV, Vladimir Petrovich; VASILENKO, Raisa Nikolaevna; Dmitry Aleksandrovich DOLGIKH;
KIRPICHNIKOV, Mikhail Petrovich; NAVOLOTSKAYA, Elena Vitalievna; KORPELA, Timo Kalevi

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)) (Appln. encl. W0 98/52594)
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(3)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

- 1.) International Preliminary Examination Report (PCT/IPEA/409) w/Amended Claims
- 2.) Six (6) Sheets of Formal Drawings

U.S. APPLICATION NO (if known, see 37 CFR 1.5)		INTERNATIONAL APPLICATION NO		ATTORNEY'S DOCKET NUMBER	
09/424080		PCT/FI98/00418		933-149PCT	

<p>17. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p>BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$970.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO. \$840.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO. \$760.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00</p> <p>ENTER APPROPRIATE BASIC FEE AMOUNT =</p> <p>Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).</p> <table border="1" style="width:100%; border-collapse: collapse;"> <tr> <th style="width: 20%;">CLAIMS</th> <th style="width: 20%;">NUMBER FILED</th> <th style="width: 20%;">NUMBER EXTRA</th> <th style="width: 20%;">RATE</th> <th style="width: 20%;"></th> <th style="width: 20%;"></th> </tr> <tr> <td>Total Claims</td> <td>4 - 20 =</td> <td>-----</td> <td>X \$18.00</td> <td>\$</td> <td>-----</td> </tr> <tr> <td>Independent Claims</td> <td>1 - 3 =</td> <td>-----</td> <td>X \$78.00</td> <td>\$</td> <td>-----</td> </tr> <tr> <td colspan="4">MULTIPLE DEPENDENT CLAIM(S) (if applicable) Yes</td> <td>+ \$260.00</td> <td>\$ 260.00</td> </tr> <tr> <td colspan="4" style="text-align: right;">TOTAL OF ABOVE CALCULATIONS =</td> <td>\$</td> <td>1360.00</td> </tr> </table> <p>Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).</p> <p style="text-align: right;">SUBTOTAL =</p> <p>Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).</p> <p style="text-align: right;">TOTAL NATIONAL FEE =</p> <p>Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +</p> <p style="text-align: right;">TOTAL FEES ENCLOSED =</p> <table border="1" style="width:100%; border-collapse: collapse;"> <tr> <td style="width: 60%;"></td> <td style="width: 20%; text-align: center;">Amount to be: refunded</td> <td style="width: 20%; text-align: center;">\$</td> </tr> <tr> <td></td> <td style="text-align: center;">charged</td> <td style="text-align: center;">\$</td> </tr> </table>	CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE			Total Claims	4 - 20 =	-----	X \$18.00	\$	-----	Independent Claims	1 - 3 =	-----	X \$78.00	\$	-----	MULTIPLE DEPENDENT CLAIM(S) (if applicable) Yes				+ \$260.00	\$ 260.00	TOTAL OF ABOVE CALCULATIONS =				\$	1360.00		Amount to be: refunded	\$		charged	\$	<table border="1" style="width:100%; border-collapse: collapse;"> <tr> <th style="width: 50%;">CALCULATIONS</th> <th style="width: 50%;">PTO USE ONLY</th> </tr> <tr> <td style="height: 150px;"></td> <td></td> </tr> </table>	CALCULATIONS	PTO USE ONLY		
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a. ☒ A check in the amount of \$ 1360.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account. No. _____ in the amount of \$ _____ to cover the above fees.
 A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
 overpayment to Deposit Account No. 02-2448.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Send all correspondence to:
Birch, Stewart, Kolasch & Birch, LLP
P.O. Box 747
Falls Church, VA 22040-0747
(703)205-8000


 SIGNATURE
 for MURPHY, GERALD M., JR.
 NAME
 #28,977
 REGISTRATION NUMBER

/GMM/sas Nov. 19, 1999

SMALL ENTITY DECLARATION

09/424080

APPLICANT OR PATENTEE ZAVIALOV, Vladimir, et al.SERIAL NO. PCT/FI98/00418☐ PATENT NO.ATTORNEY'S
DOCKET NO.933-149P(Check one
of blocks
1 or 2.)1. ☐ FILED OR ISSUED2. ☐ SUBMITTED HEREWITHFOR "Compositions for Enhancing Immunosuppressants' Pharmaceutical Activities"

(Insert Title)

I(we) hereby declare that I(we) am(are) entitled to the benefit of small entity status with respect to the above-identified application or patent for purposes of paying reduced fees under 35 USC 41(a) & (b) to the U.S. Patent and Trademark Office.

☒ A. INDEPENDENT INVENTOR

I(we) qualify as (an) independent inventor(s) as defined in 37 CFR 1.9(c).

☐ B. INDIVIDUAL NON-INVENTOR

I would qualify as an independent inventor as defined in 37 CFR 1.9(c) if I had made the invention.

☐ C. SMALL BUSINESS CONCERN

I am ☐ THE OWNER ☐ AN OFFICIAL of the small business concern identified below and am empowered to act on behalf of the concern. The concern qualifies under 37 CFR 1.9(d) and 13 CFR 121.3-18. Rights under contract or law have been conveyed to and remain with the concern and are exclusive unless a checkmark is placed here ☐. All other rights belong to small entities as defined in 37 CFR 1.9.

☐ D. NON-PROFIT ORGANIZATION

I am an official empowered to act on behalf of the non-profit organization identified below. The organization qualifies under 37 CFR 1.9(e), sub-section: ☐ (1) ☐ (2) ☐ (3) ☐ (4). Rights under contract or law have been conveyed to and remain with the organization and are exclusive unless a checkmark is placed here ☐. All other rights belong to small entities as defined in 37 CFR 1.9.

I(we) acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I(we) declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

A. INDEPENDENT INVENTOR(S) B. INDIVIDUAL NON-INVENTOR(S)

KIRPICHNIKOV, Mikhail

Name

Signature

Date

14.01.2000NAVOLOTSKAYA, Elena

Name

Signature

Date

now14.01.2000KORPELA, Timo

Name

Signature

Date

Timo Korpele14.01.00

C. BUSINESS CONCERN D. NON-PROFIT ORGANIZATION

Name of Concern or Organization

Address

By

Name of Person Signing

Signature

Title

Date

SMALL ENTITY DECLARATION

09/424080

APPLICANT OR PATENTEE ZAVIALOV, Vladimir, et al.SERIAL NO. PCT/FI98/00418☐ PATENT NO.

ATTORNEY'S

DOCKET NO.

933-149P1. ☐ FILED OR ISSUED2. ☐ SUBMITTED HERewithFOR "Compositions for Enhancing Immunosuppressants' Pharmaceutical Activities"

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I(we) declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

A. INDEPENDENT INVENTOR(S) B. INDIVIDUAL NON-INVENTOR(S)

ZAVIALOV, Vladimir

Name

Signature *Zavialov*

Date

14.01.2000VASILENKO, Raisa

Name

Signature *Raisa*

Date

14.01.2000DOLGIKH, Dmitry

Name

Signature *D. Dolikh*

Date

14.01.2000

C. BUSINESS CONCERN D. NON-PROFIT ORGANIZATION

Name of Concern or Organization

Address

By

Name of Person Signing

Signature

Title

Date

001760 00012100

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420 Rec'd PCT/PTO 19 NOV 1999

PATENT
933-149PCT

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: ZAVIALOV, Vladimir Petrovich et al
Int'l. Appl. No.: PCT/FI98/00418
Appl. No.: New Group: Unknown
Filed: November 19, 1999 Examiner: Unknown
For: COMPOSITIONS FOR ENHANCING IMMUNOSUPPRESSANTS
PHARMACEUTICAL ACTIVITIES

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION

Assistant Commissioner for Patents
Washington, DC 20231

November 19, 1999

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Before line 1, insert --This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/FI98/00418 which has an International filing date of May 18, 1998, which designated the United States of America.--

REMARKS

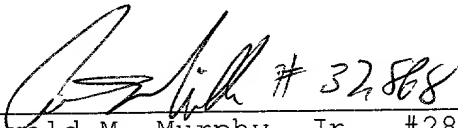
The specification has been amended to provide a cross-reference to the previously filed International Application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By

 # 32868
Gerald M. Murphy, Jr., #28,977

P.O. Box 747
Falls Church, VA 22040-0747
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GMM/sas

(Rev. 03/30/99)

COMPOSITIONS FOR ENHANCING IMMUNOSUPPRESSANTS'
PHARMACEUTICAL ACTIVITIES

5 FIELD OF INVENTION

This invention is related to human or animal medicine and more specifically to improving the therapeutic effects of common immunosuppressive, anti-inflammatory and/or antiparasitic drugs where the effective ingredients contain cyclosporins, FK506 or rapamycin. This invention provides new compositions for efficient amplification of the activities of the said components to decrease their therapeutic dose to avoid their side effects.

15 BACKGROUND OF INVENTION

Cyclosporins are cyclic peptides composed of eleven amino acid residues which include previously unknown N-methylated amino acid residues. Cyclosporins are commercially important products isolated from the culture broths of the fungal species *Tolypocladium*. Cyclosporins are applied to prolong survival of allogenic transplants involving skin, kidney, pancreas, bone marrow, small intestine and lung. The efficacy of cyclosporins is presently known to be due to the specific and reversible inhibition of immunocompetent cells, primarily T-helper cells. Unfortunately, cyclosporins' systemic use have been associated with a high incidence of renal toxicity (kidney failure), some cases of hepatotoxicity, increased incidence of lymphoid tumors and increased incidence of opportunistic infections. The side effects of cyclosporins are so severe and so common that they limit their applications to life-threatening or severe sight-threatening diseases.

Cyclosporins, like FK506 and other related molecules, bind specifically to certain physiologically important molecular species in tissues and blood circulation (Denesyuk A. et al., 1993, Biochem. Biophys. Res. Commun. 197, pp. 1438-1442; Denesyuk A. et al., 1993, Biochem. Biophys. Res. Commun. 194, pp. 280-286; Denesyuk A. et al., 1993, Biochem. Biophys. Res. Commun. 192, pp. 912-917). After identification of cyclosporin

A, other cyclosporins, B, C, D, E, and G, containing minor differences in the molecular structure of cyclosporins have been reported. Many of these cyclosporins exhibit pharmaceutical utility related to cyclosporin A and the literature involves abundantly examples and suggestions for their use as therapeutic agents.

5

Cyclosporins were first proposed for be used as an antifungal agents, but their immunosuppressive action was found to be of more importance. Cyclosporins specifically and reversibly inhibit immunocompetent cells, primarily T-helper cells, by specific interactions with peptidyl-prolyl *cis-trans* isomerase (PPIase; see, Schreiber S. and Crabtree G., 1992, Immunol. Today 13, pp. 136-142; Zav'yalov V. et al., 1995, Acta Pathol. Microbiol. Immunol. Scand. 103, pp. 401-415). These multifunctional PPIase proteins may also serve as ATP-independent molecular chaperones facilitating the folding of target proteins and assembly of multisubunit protein complexes (Schmid F. et al., 1993, Adv. Protein Chem. 46, pp. 25-65), but their primary function is the binding of the immunosuppressive drugs cyclosporin A, FK506 and rapamycin. The PPIases with such activity were termed immunophilins (ImP's). These protein receptors fall into two major groups for their structure: the cyclosporin binding proteins, also termed cyclophilins (CyPs), and the FK506 binding proteins, termed FKBP's, some of the latter preferentially binding rapamycin (Trandini C. et al., 1992, FASEB J. 6, pp. 3410-3420; Liu J., 1993, Immunol. Today 14, pp. 290-295). It has been found that Ca^{2+} and calmodulin-dependent protein phosphatase, calcineurin (CaN), binds with high affinity to biologically active ImP-drug complexes. The experimental data suggest that the cytoplasmic NF-AT protein (NF-ATc) is a substrate, either direct or indirect, via a phosphatase cascade for CaN. NF-AT is a complex protein made of at least two subunits. One subunit of NF-AT (NF-ATp), a DNA-binding phosphoprotein, is a substrate for CaN *in vitro* and interacts with Fos and Jun proteins in the nucleus of activated T cells (Jain J. et al., 1993, Nature 365, pp. 352-355). Dephosphorylation by CaN might directly induce nuclear translocation of NF-ATc. The active form of this protein interacts with functional sequences within the IL-2 enhancer. According to a hypothesis, the complexes cyclosporin A-CyP or FK506-FKBP-12 block nuclear translocation of NF-ATc and activation of IL-2 gene expression due to the inhibition of CaN activity.

30

Cyclosporins' immunosuppressive properties have led to their wide routine usage in immune system-related diseases, as described, for example, in Finnish patent publication 900604. Correspondingly, the number of patents for these purposes on various aspects of cyclosporins is large. In addition, eye medicine has benefited from cyclosporins. The U.S. Patent No. 4, 649, 047 describes a method for the treatment of phacoanaphylactic endophthalmitis and uveitis in the anterior or posterior segment of an eye wherein cyclosporin is topically administered to the eye. In other ophthalmic applications, cyclosporin has been used topically only for the treatment of external (e.g., corneal) eye diseases. BenEzra et al., 1986, Amer. J. Ophthalmol. 101, pp. 278-282 described the effect of 2% cyclosporin eyedrops on severe vernal keratoconjunctivitis which is a seasonal allergic disorder unrelated to tear deficiency. Hunter et al., 1981, Clin. Exp. Immunol. 45, pp. 173-177 described the topical administration of cyclosporin in a rabbit model of corneal graft rejection with positive results. Boisjoly et al., 1984, Arch. Ophthalmol. 102, pp. 1804-1807 reported that topical application of cyclosporin had a beneficial prophylactic effect towards the treatment of severe herpetic stromal keratitis. Mosteller et al., 1984, Investigative Ophthalmol. Supp. 25, pp. 3-38 treated corneal allograft rejection in rabbits by applying a single dose of a 10% cyclosporin A ointment in the lower cul-de-sac of the eyelids. Cyclosporins have also been used systemically in other ophthalmic applications, where the disease being treated is not limited to the eye surface. For example, Nussenblatt et al., 1983, Amer. J. Ophthalmol. 96, pp. 275-282 reported clinical improvement in some patients with noninfectious posterior uveitis following systemic treatment with cyclosporin.

Cyclosporins are primarily administered orally or by injection. Unfortunately, cyclosporins used systemically have been associated with a high incidence of renal toxicity (kidney failure), some cases of hepatotoxicity, increased incidence of lymphoid tumors and increased incidence of opportunistic infections. Cyclosporin are only slightly less toxic than other immunosuppressive agents such as cytoxan or aziothioprine. The systemic side effects of cyclosporins are so severe and so common that they limit cyclosporins use to life-threatening or in some cases severe sight-threatening diseases.

As described in U.S. Patent No. 4, 117, 118 1978 (Harri et al.), cyclosporin is readily soluble in most of the usual organic solvents and practically insoluble in petroleum ether and water. Poor solubility of cyclosporins makes absorption in intestine with oral administration as well as actions in intravenous applications variable from patient to patient. If rather high and constant level of cyclosporins are required in the blood circulation, administration of cyclosporins is complex. As distributed by Sandoz Ltd. under the tradename Sandimmune, cyclosporin for oral administration is dissolved in olive oil for further dilution with food and in polyoxyethylated castor oil and ethanol for intravenous injection. The release of cyclosporins from the carrier substances is patient-dependent where either the dose is not therapeutically effective or it is toxic. As stated, prolonged systemic administration of cyclosporins associated with transplantation and related uses creates risks for other diseases including cancers. To be able to use considerably lower than toxic level of effective cyclosporin doses is therefore of key importance.

Recently, the attention was brought to the long-neglected role of type I interferons (IFN- α , β , γ , δ) in the modulation of immune response (Belardelli F. and Gresser I., 1996, Immunol. Today 17, pp. 369-372). The immunomodulating activity of IFN- α , β , γ , δ is very complex and includes:

- inhibition of lymphocyte proliferation after stimulation with lectins or allogenic cells;
- prolongation of skin grafts;
- inhibition of delayed type of hypersensitivity;
- differentiation towards a Th1 type of immune response;
- stimulation *in vivo* of long-lived antigen-specific memory CD8+ cell cytotoxicity;
- inhibition of suppressor T cells;
- enhancement of natural killer (NK) cell cytotoxicity;
- enhancement of major histocompatibility complex (MHC) class I antigens expression.

First three immunomodulating properties of type I IFNs (inhibition of lymphocyte proliferation after stimulation with lectins or allogenic cells, prolongation of skin grafts, and inhibition of delayed type of hypersensitivity) are useful for amplification of

immunosuppressants' (cyclosporins, FK506 and rapamycin) activity. But other immunomodulating properties of type I IFNs (differentiation towards a Th1 type of immune response; stimulation *in vivo* of long-lived antigen-specific memory CD8⁺ cell cytotoxicity; inhibition of suppressor T cells; enhancement of NK cell cytotoxicity; enhancement of MHC class I antigens expression) can induce an opposite effect. Indeed, it was observed that a monoclonal antibody directed against the extracellular domain of the human (h)IFN- α receptor, which inhibits both the binding and biological activity of all the type I IFNs tested, exerted a dose-dependent inhibition of the mixed lymphocyte reaction and induced permanent survival of skin allografts in MHC-divergent Cynomolgus monkeys treated with a subeffective dose of cyclosporin A (Tovey, M.G. et al, 1996, J. Leukocyte Biol. 59, pp. 512-517).

In the present invention we suprisingly observed that the physiological effects of immunosuppressants (cyclosporins, FK506 and rapamycin) can be dramatically increased by the simultaneous administration of any one of the immunosuppressants and the peptide corresponding to the high-affinity binding/anti-lymphoproliferative site of IFN- α or the recombinant protein having the amino acid sequence corresponding to the said site. Peptides are specially favourable additives since they do not cause harmful symptoms and the activity quenching due to the generation of antibodies against larger polypeptides such as natural interferons.

Long time ago there was discovered the primary structure homology of hIFNs- α /b and thymus hormone - thymosin α_1 (TMA₁) (Zav'yalov V. and Denesyuk A., 1984, Doklady Akademii Nauk SSSR 275, pp. 242-246). Comparison of the primary structures of proTMA and hIFNs- α /b reveals a homology of the prohormone with the IFNs' sites making up the C-terminal helices D and E, however, the highest homology is observed for the C-terminal part of helix D and the N-terminal part of loop DE (Zav'yalov V. et al., 1989, Immun. Lett. 22, pp. 173-182; Zav'yalov V. et al., 1990, Biochim. Biophys. Acta 1041, pp.178-185). One of the synthesized peptides (α -peptoferon) overlapping the TMA₁-like sequence of hIFN- α 2, effectively competed with hIFN- α 2, TMA₁, proTMA for common receptors on mouse thymocytes (i.e. the K_i of recombinant (r)hIFN- α 2 binding by α -peptoferon is equal to about 10^{-12} M) (Zav'yalov V. et al., 1991, FEBS Lett. 278,

pp. 187-189; Zav'yalov V. et al., 1995, Molec. Immun. 32, pp. 425-431). Recently the first example of successful grafting of hIFN- α 2's TMA₁-like site to the design *de novo* protein albeferon was described (Dolgikh D. et al., 1996, Protein Engin. 9, pp. 195-201). The IFN- α 2 fragment corresponded to the TMA₁-like sequence 130-137 was inserted into the N-termini of an albebetin molecule just after the initiatory Met residue. The chimeric protein (albeferon) was expressed in a wheat germ cell-free translation system and tested for its compactness and stability. It has been shown that albeferon is practically as compact as natural proteins of corresponding molecular weight and possesses high stability toward the urea-induced unfolding. To testify the affinity of albeferon to murine thymocyte receptor, the protein inhibitory effect on the binding of radiolabeled [¹²⁵I] a-peptoferon to the receptors has been studied. The albeferon competitive inhibition coefficient (IC₅₀) and the calculated inhibition constant (K_i) are very close to that of rhIFN- α 2. It was demonstrated that cell surface binding characteristics correlate with consensus type I IFN enhanced anti-lymphoproliferative activity on the human periferal polymorphonuclear cells (Klein S. et al., 1996, J. Interferon and Cytokine Res. 16, pp. 1-6; Dhib-Jalbut S. et al., 1996, J. Interferon and Cytokine Res. 16, pp. 195-200).

The present invention includes the compositions for efficient amplification of immunosuppressive activity of cyclosporins, FK506 or rapamycin by peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of IFNs- α , β , γ , δ or recombinant proteins having the amino acid sequences corresponding to the said site to decrease therapeutic dose of the both groups of compounds, and as the consequence to avoid their undesirable side effects during organ and tissue transplantation, and treatment of different diseases.

SUMMARY OF THE INVENTION

The present invention provides the compositions for efficient amplification of immunosuppressive activity of cyclosporins, FK506 or rapamycin to decrease therapeutical dose of immunosuppressants, and as the consequence to avoid their

undesirable side effects during organ and tissue transplantation, and treatment of different diseases.

These compositions include cyclosporins, FK506 or rapamycin and biologically active peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of IFNs-
5 a,b,w,t, or recombinant proteins having the amino acid sequences corresponding to the said site.

BRIEF DESCRIPTION OF THE DRAWINGS

10 FIG. 1 Effect of rhIFN-a2 and of the peptide corresponding to the high-affinity binding/anti-lymphoproliferative site of hIFN-a2 (a-peptoferon) on mitogen (PHA)-driven proliferation of human peripheral polymorphonuclear cells.

15 FIG. 2 Effect of rhIFN-a2 and of the peptide corresponding to the high-affinity binding/anti-lymphoproliferative site of hIFN-a2 (a-peptoferon) on the proliferation of an IFN-sensitive human T-lymphoblastoid cell line MT-4.

20 FIG. 3 Effect of rhIFN-a2, albeferon and albebetin on mitogen (PHA)-driven proliferation of human peripheral polymorphonuclear cells.

FIG. 4 Effect of rhIFN-a2, albeferon and albebetin on the proliferation of an IFN-sensitive human T-lymphoblastoid cell line MT-4.

25 FIG. 5 Effect of rhIFN-a2 and of the peptide corresponding to the high-affinity binding/anti-lymphoproliferative site of hIFN-a2 (a-peptoferon) on mitogen (PHA)-driven proliferation of human peripheral polymorphonuclear cells in the presence of 20 mM cyclosporin A in cultural medium.

30 FIG. 6 Effect of albeferon on mitogen (PHA)-driven proliferation of human peripheral polymorphonuclear cells in the presence of 10 mM cyclosporin A in cultural medium.

DETAILED DESCRIPTION OF THE INVENTION

Cyclosporins, FK506 and rapamycin are widely used commercially important drugs for treatment of various diseases of human and higher animals. However, the use of these drugs have severe drawbacks for their toxicity and narrow effective dose range. This forms the basis of either developing other more effective and less toxic related drugs or increasing their activity by other ingredients. The latter has been attempted only by making drug formulations which provide either more effective absorption in intestine or prolonged delivery in intestine or in intravenous administration.

In this invention, there was surprisingly observed that certain favourable physiological effects of cyclosporins, FK506 and rapamycin on human cells can be amplified by biologically active peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of IFNs-a,b,w,t or recombinant proteins having the amino acid sequences corresponding to the said site. The peptides and recombinant proteins mentioned above can be readily produced by widely available synthetic or recombinant techniques. The present invention provides definite improvement of compositions for drugs based on cyclosporins, FK506 or rapamycin aimed for human and higher animals. These compositions include the immunomodulators and biologically active peptides corresponding to the high-affinity binding/anti-proliferative site of IFNs-a,b,w,t, or recombinant proteins having the amino acid sequences corresponding to the said site. Although all the potentially bioactive peptides or the recombinant proteins containing the amino acid sequences of the peptides were not possible to test experimentally here with the immunomodulator drugs (cyclosporins, FK506 and rapamycin) such structures were revealed by extensive comparisons of available amino acid sequences of interferons from different species by computer and molecular modelling techniques taking into account the experimental data on the localisation of the high-affinity binding/anti-lymphoproliferative site of IFNs-a,b,w,t and the experimental data on the competition of type I IFNs for the common receptors. The peptides according to this invention have the advantage of not give rise to antibodies like large polypeptides and natural interferons and thus peptides as such or bound to carrier molecules can be used for long periods.

In the previous studies (Zav'yalov, V. et al., 1991, FEBS Lett. 278, pp. 187-189; Zav'yalov V. et al., 1995, Molec. Immun. 32, pp. 425-431) it was demonstrated that only octapeptide corresponding to the 130-137 amino acid residues of hIFN-a2 had the same or higher affinity to the specific receptors in comparison with recombinant hIFN-a2. It is well-known that all type I IFNs compete for the common receptors and can induce the common type I IFN activities. Therefore, it is reasonable to assume that in the process of natural selection the changes in the amino acid sequence of the high-affinity binding/anti-lymphoproliferative site of IFNs-a,b,w,t were selected not to abolish the biological activities of the site. Consequently, all type I natural and recombinant IFNs as well as peptides corresponding to their high-affinity binding/anti-lymphoproliferative site, and recombinant proteins having the amino acid sequences corresponding to the said site might reproduce the anti-proliferative activity and amplify immunosuppressive activity of cyclosporins.

For the testing of synergism of cyclosporin, FK506 or rapamycin and peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of IFNs-a,b,w,t or recombinant proteins having the amino acid sequences corresponding to the said site we employed the classic anti-lymphoproliferative test system with human peripheral polymorphonuclear cells and human T-lymphoblastoid cell line MT-4. The cell culture conditions and the conditions of the humoral cells in blood circulation are closely related. In fact, in cell cultures, which are commonly used for testing of potential drugs, the conditions are strictly maintained similar to the blood circulation as to the temperature, pH, buffer, minerals, CO₂ and O₂ partial pressures and so on. On the other hand, in this special case the target cells of cyclosporins or the other immunosuppressants and the peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of IFNs-a,b,w,t or recombinant proteins having the amino acid sequences corresponding to the said site being used as the drugs exist specifically in the blood circulation in very equal conditions to the cell cultures. Thus, it is highly predictable that the drug compositions of the present invention can be used as medical drugs for purposes previously used for immunosuppressants alone as the active ingredient in the drug formulations.

While the main experimental proof of the present invention lies on the use of peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of IFNs-a,b,w,t or

recombinant proteins having the amino acid sequences corresponding to the said site with cyclosporins, also FK506 and rapamycin can be applied with related amplification effect as with cyclosporins. Although the present invention describes effects of peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of IFNs-a,b,w,t or recombinant proteins having the amino acid sequences corresponding to the said site on immunosuppressants such as cyclosporins, FK506 and rapamycin, it is evident that the peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of IFNs-a,b,w,t or recombinant proteins having the amino acid sequences corresponding to the said site will increase equally well the activities of any other immunosuppressant.

Example 1.

Ninety five ml of blood were taken from each of 10 volunteer donors, and 5 ml of 3-5% EDTA were added to the blood immediately and mixed carefully to prevent coagulation. The blood was then mixed with the equal volume of medium 199 or with buffered physiological salt solution. Ficoll-Paque (Pharmacia) was poured into plastic centrifuge test-tubes with caps (Costar) (3 ml of Ficoll-Paque per each test-tube, volume of a test-tube - 15 ml, diameter - 13 mm at room temperature, and then 6-7 ml of diluted blood were carefully layered on top). The test-tubes were centrifuged at 20 °C for 40 minutes, at 400 g, with the brake of centrifuge being switched off. The mononuclear cells formed a layer on the border of the liquid division and the cells were collected with a Pasteur pipette into a centrifuge test-tube with 5 ml of RPMI 1640, containing 2% of inactivated fetal serum and penicillin (100 units/ml) with streptomycin (100 units/ml). Then the cells were washed with physiological buffer solution (PBS) three times with centrifuging at 400 g for 5 minutes at the temperature of +8 °C. When the concentration of the cells was $2.5 \cdot 10^6$ cells/ml, they were placed in RPMI 1640 medium, containing 5% of fetal serum (heated for 30 minutes at 56 °C), 100 units/ml of penicillin, 100 units/ml of streptomycin, 1 ml 200 mM of L-glutamine per 100 ml of serum. All the reagents were produced by Gibco. The cells were dropped into a 96-well plate (100 ml per each plate) with mitogen - phytohemagglutinin (PHA) (final concentration - 2 mg/ml), the preparations under study and immunosuppressants (cyclosporin A, FK506 or rapamycin) in concentration - 5-20

mM) were pipetted into the tubes beforehand. The plate was placed in a CO₂-incubator at 5% CO₂ with humid atmosphere for 72 hours. 12-18 hours before the completion of the incubation 1 mCi of [³H]-thymidine was added into each well. Then the cells were washed on a semiautomatic harvester (Titertec Flow Lab.) using filters (Whatman). Each filter was carefully transferred into bottles, containing 5 ml of scintillation liquid (Sigma). The number of disintegrations per minute was counted using a Minibeta counter (LKB). The stimulation index was determined by arithmetical mean of at least three parallel measurements \pm SD to the corresponding control.

- Fig. 1 displays, respectively, the effect of rhIFN-a2 and of the peptide corresponding to the high-affinity binding/anti-lymphoproliferative site of hIFN-a2 (a-peptoferon) on mitogen (PHA)-driven proliferation of human peripheral polymorphonuclear cells. PHA induced strong lymphoproliferative response that was inhibited by rhIFN-a2 and a-peptoferon in a dose-dependent fashion. rhIFN-a2 and a-peptoferon demonstrated comparable effects. rhIFN-g had either no effect or a minimal enhancing effect on the lymphoproliferative response. (1) rhIFN-a2; (2) a-peptoferon; (3) control: PHA; (4) control: cultural medium.

Example 2.

- Fig. 2 shows the effect of rhIFN-a2 and of the peptide corresponding to the high-affinity binding/anti-lymphoproliferative site of hIFN-a2 (a-peptoferon) on the proliferation of an IFN-sensitive human T-lymphoblastoid cell line MT-4. The proliferation of human T-lymphoblastoid cell line MT-4 was inhibited by rhIFN-a2 and a-peptoferon in a dose-dependent fashion. rhIFN-a2 and a-peptoferon demonstrated comparable effects. Similar methods as in Example 1 were employed (1) rhIFN-a2; (2) a-peptoferon.

Example 3.

- Fig. 3 displays the effect of rhIFN-a2 and the designed *de novo* chimeric protein albeferon containing the TMA₁-like sequence 130-137 of the hIFN-a2 inserted into the N-termini of the protein just after the initiatory Met residue (2), and the designed *de novo* protein albebetin without the hIFN-a2 fragment (3) on PHA-driven proliferation of human peripheral polymorphonuclear cells. PHA induced strong lymphoproliferative response

that was inhibited by rhIFN-a2 and albeferon in a dose-dependent fashion. rhIFN-a2 and albeferon demonstrated comparable effects. Albebetin had no effect on the lymphoproliferative response. The results show that the hIFN-a2 fragment corresponded to the TMA₁-like sequence 130-137 is responsible for the anti-lymphoproliferative effect of rhIFN-a2. Similar methods as in Example 1 were employed, (1) rhIFN-a2; (2) albeferon; (3) albebetin; (4) control: cultural medium; (5) control: PHA

Example 4.

Fig. 4 demonstrates the effect of rhIFN-a2, albeferon and albebetin on the proliferation of an IFN-sensitive human T-lymphoblastoid cell line MT-4. The proliferation of human T-lymphoblastoid cell line MT-4 was inhibited by rhIFN-a2 and albeferon in a dose-dependent fashion. In this experiment albeferon had higher activity than the sample of rhIFN-a2 used but comparable with other data on rhIFN-a2 (see Figs. 1-2 and the data described in: Dhib-Jalbut S. et al., 1996, J. Interferon and Cytokine Res. 16, pp. 195-200). Albebetin had no effect on the lymphoproliferative response. The results show that the hIFN-a2 fragment corresponded to the TMA₁-like sequence 130-137 is responsible for the anti-lymphoproliferative effect of rhIFN-a2. (1) rhIFN-a2; (2) albeferon; (3) albebetin.

Example 5.

Fig. 5 displays the effect of rhIFN-a2 on PHA-driven proliferation of human peripheral polymorphonuclear cells in the presence of 20 mM cyclosporin A (CsA) in cultural medium. PHA induced strong lymphoproliferative response. The selected concentration of CsA had enhancing effect on the lymphoproliferative response. The administration of extremely low amount of rhIFN-a2 (10^{-16} M) totally abolished lymphoproliferative response induced by PHA. The comparable effect on mitogen-driven proliferation of peripheral blood human T-lymphocytes in the presence of 20 mM CsA in cultural medium was observed for the octapeptide corresponding to the high-affinity binding/anti-lymphoproliferative site of hIFN-a2 (a-peptiferon). The results demonstrate strong synergism of anti-lymphoproliferative action of CsA and rhIFN-a2 or the peptide corresponding to the high-affinity binding/anti-lymphoproliferative site of hIFN-a2 (a-peptiferon). Related results were obtained with 1-50 mM concentration of FK506 or rapamycin instead of cyclosporin A. Similar methods as in Example 1 were employed. (1)

rhIFN- α 2 and 20 mM CsA; (2) α -peptiferon and 20 mM CsA; (3) control: cultural medium; (4) control: 20 mM CsA; (5) control: PHA; (6) control: 20 mM CsA and PHA.

Example 6.

- 5 Fig. 6 displays the effect of albeferon on PHA-driven proliferation of human peripheral polymorphonuclear cells in the presence of 10 mM cyclosporin A (CsA) in cultural medium. PHA induced strong lymphoproliferative response. The selected concentration of CsA had enhancing effect on the lymphoproliferative response. The administration of extremely low amount of albeferon (10^{-12} M) totally abolished lymphoproliferative
- 10 response induced by PHA. Related results were obtained with 1-50 mM concentration of FK506 or rapamycin instead of cyclosporin A. Fig 6 also shows that the octapeptide corresponding to the high-affinity binding/anti-lymphoproliferative site of hIFN- α 2 (α -peptiferon) which was genetically immobilized on the macromolecular carrier (i.e. on the *de novo* protein albebetin) is biologically active. Similar methods as in Example 1 were
- 15 employed. (1) albeferon; (2) albeferon and 10 mM CsA; (3) control: cultural medium; (4) control: PHA; (5) control: PHA and 10 mM CsA.
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Claims

1. Any drug composition consisting of immunosuppressants cyclosporins, FK506, or rapamycin and the bioactive peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of interferons $\alpha, \beta, \omega, \tau$, or recombinant proteins carrying one or more of the sequences corresponding to the structures of the bioactive peptides corresponding to the high-affinity binding/anti-lymphoproliferative site of the said interferons for the aim of amplification of immunosuppressants' activities to decrease their therapeutic dose, and as the consequence to avoid their undesirable side effects during organ and tissue transplantation or during treatment of cancers such as lymphomas, leukemias, myelomas, adenocarcinomas, autoimmune and chronic inflammatory diseases, such as rheumatoid arthritis, myasthenia gravis, lupus erythematosus, uveitis, hyperproliferative diseases, such as psoriasis vulgaris, wherein cyclosporins, FK506 or rapamycin can be exploited.

2. Compositions according to Claim 1 consisting of immunosuppressants cyclosporins, FK506 or rapamycin with one or more of the peptides containing at least 5 identical amino acid residues in the following table:

L	T	E	K	K	Y	S	P
	R	R	R	R	H	R	R
	Q	D	N	D		D	D
	M	L	M	N		N	L
		I	S	S		S	
	E	A	E			A	
	G	I					

wherein,

each amino acid residue in the top sequence can be modified with any of the amino acid residues under it.

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3. Compositions according to Claim 1 consisting of immunosuppressants cyclosporins, FK506 or rapamycin, and recombinant proteins carrying one or more of the sequences corresponding to the peptide variation of Claim 2.

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4. Compositions according to Claim 2 consisting of immunosuppressants cyclosporins, FK506 and rapamycin, and the bioactive peptides wherein the active peptides are genetically or chemically modified or genetically or chemically or physically bound to a small-molecular or macromolecular substance for the aim of increasing the stabilities of the peptides in physiological conditions or for regulating the bioavailability of the said peptides.

10

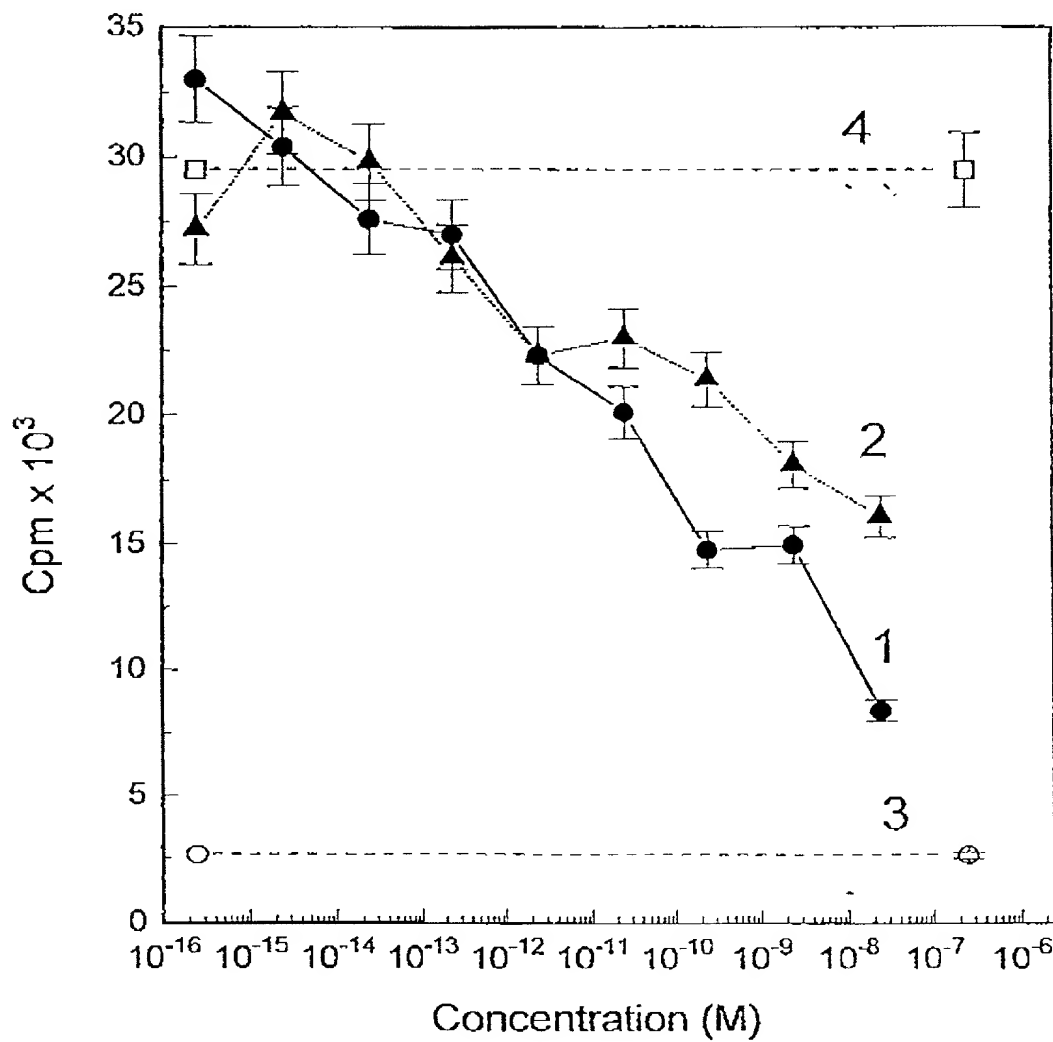


Fig.1

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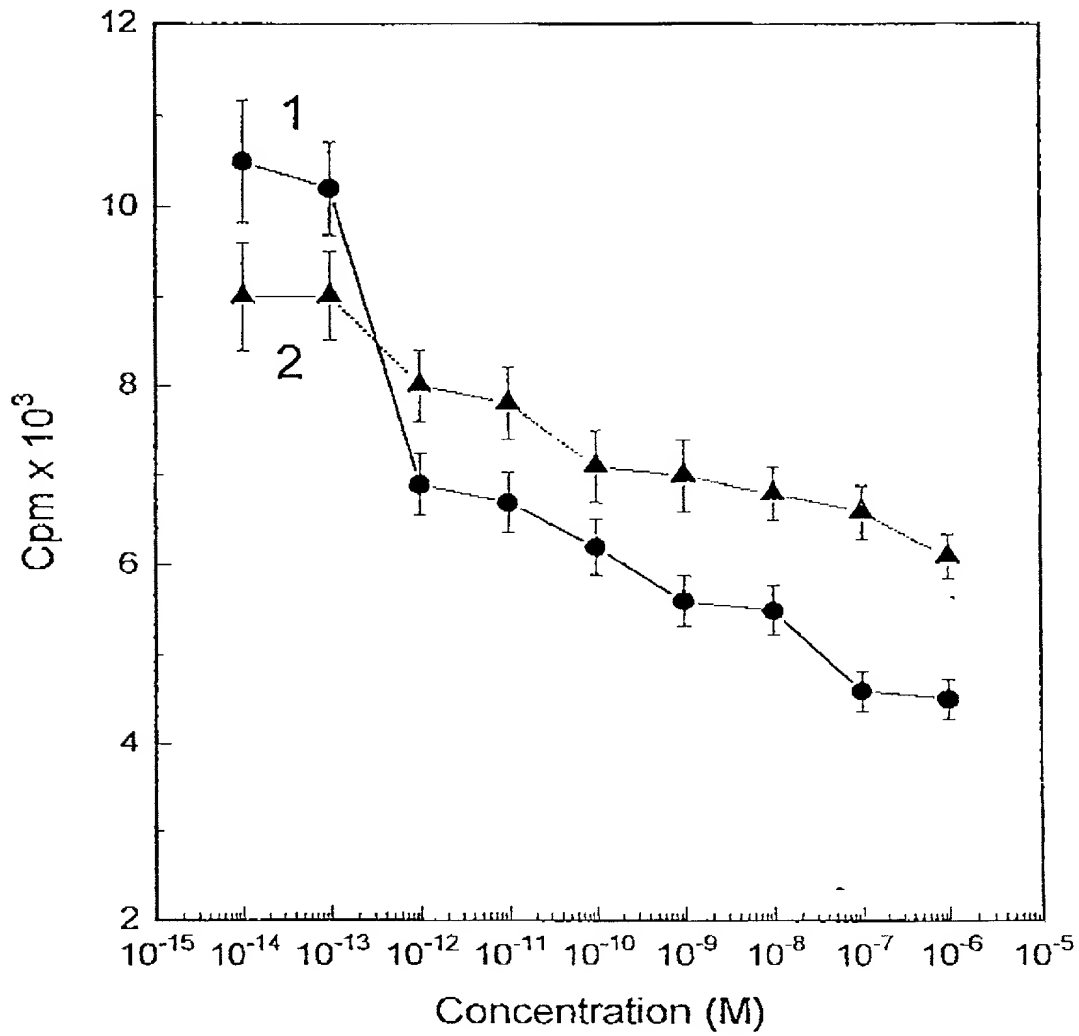


Fig.2

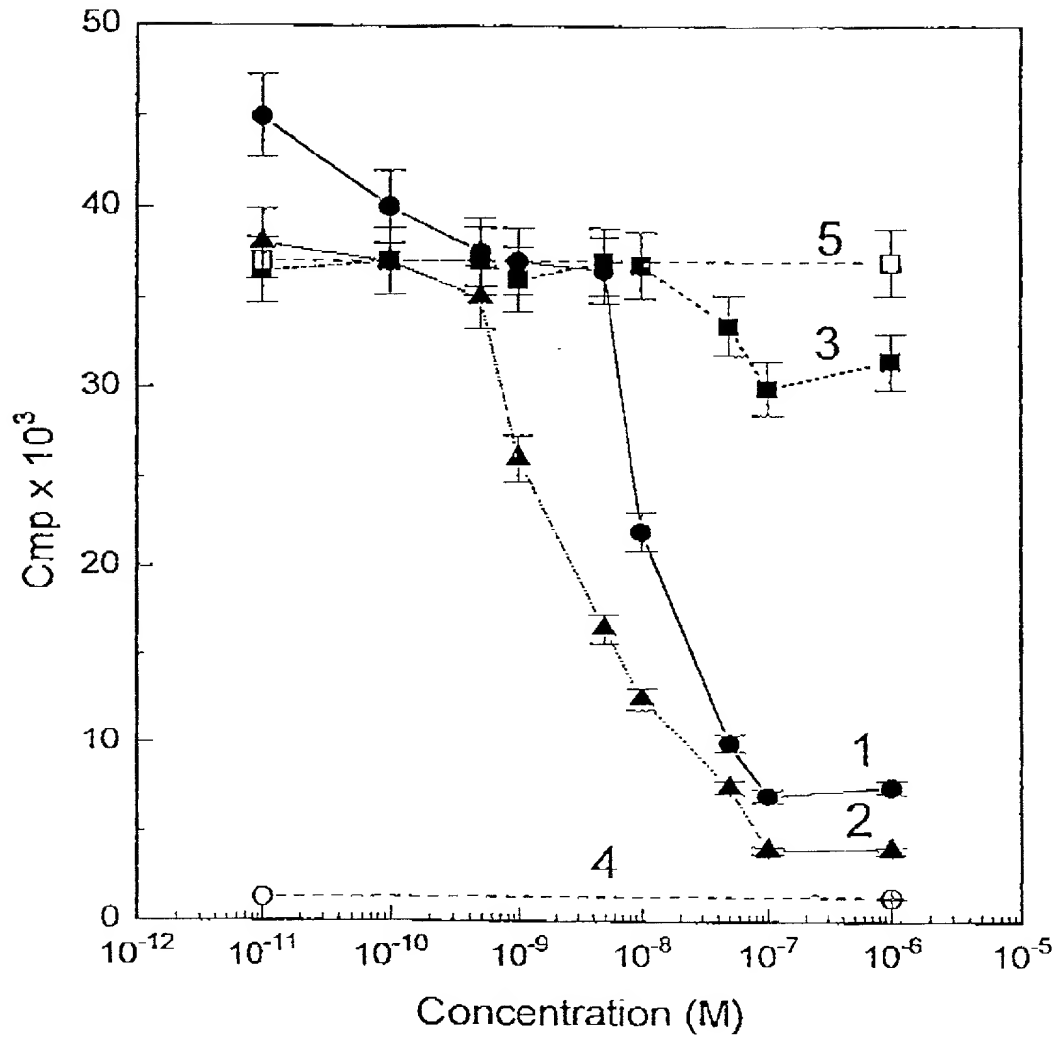


Fig.3

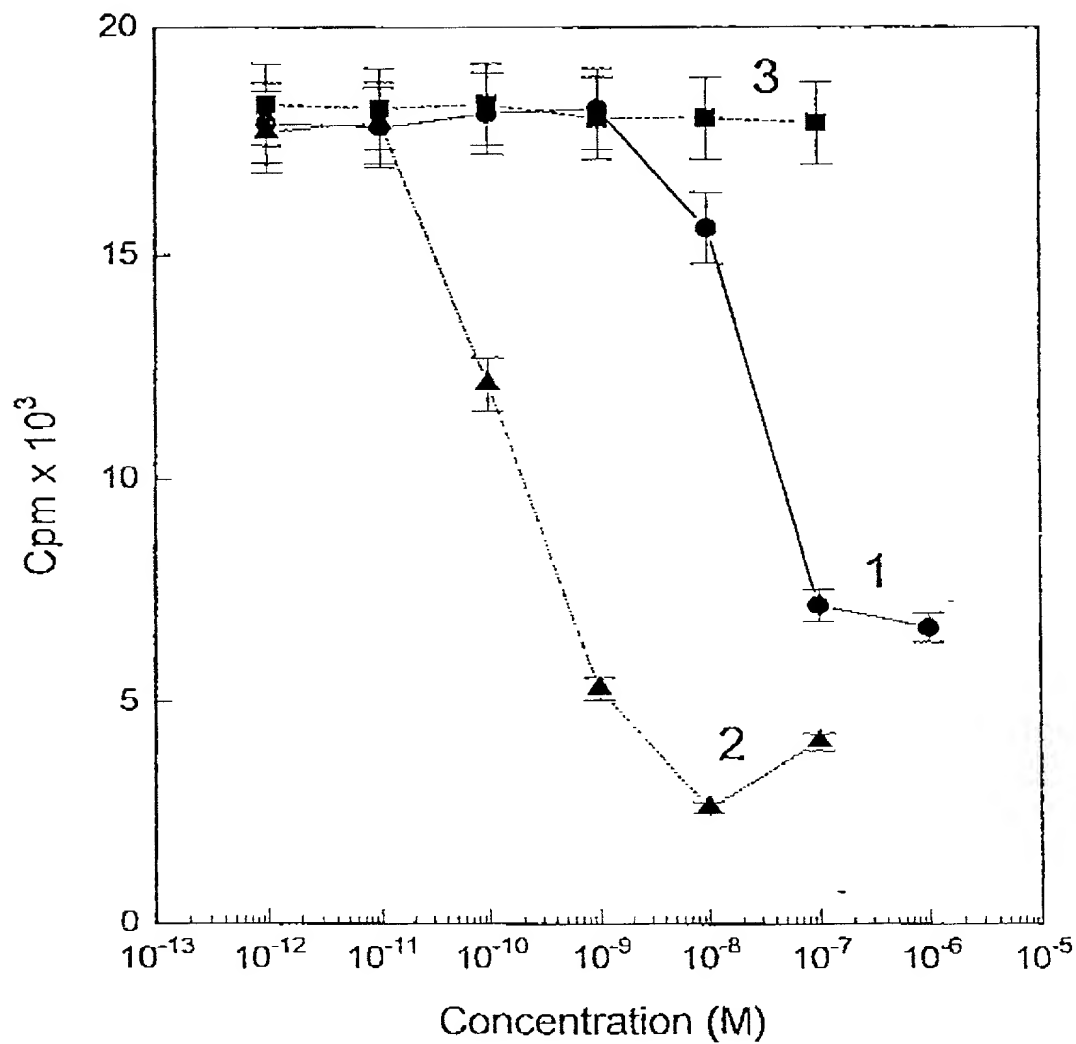


Fig. 4

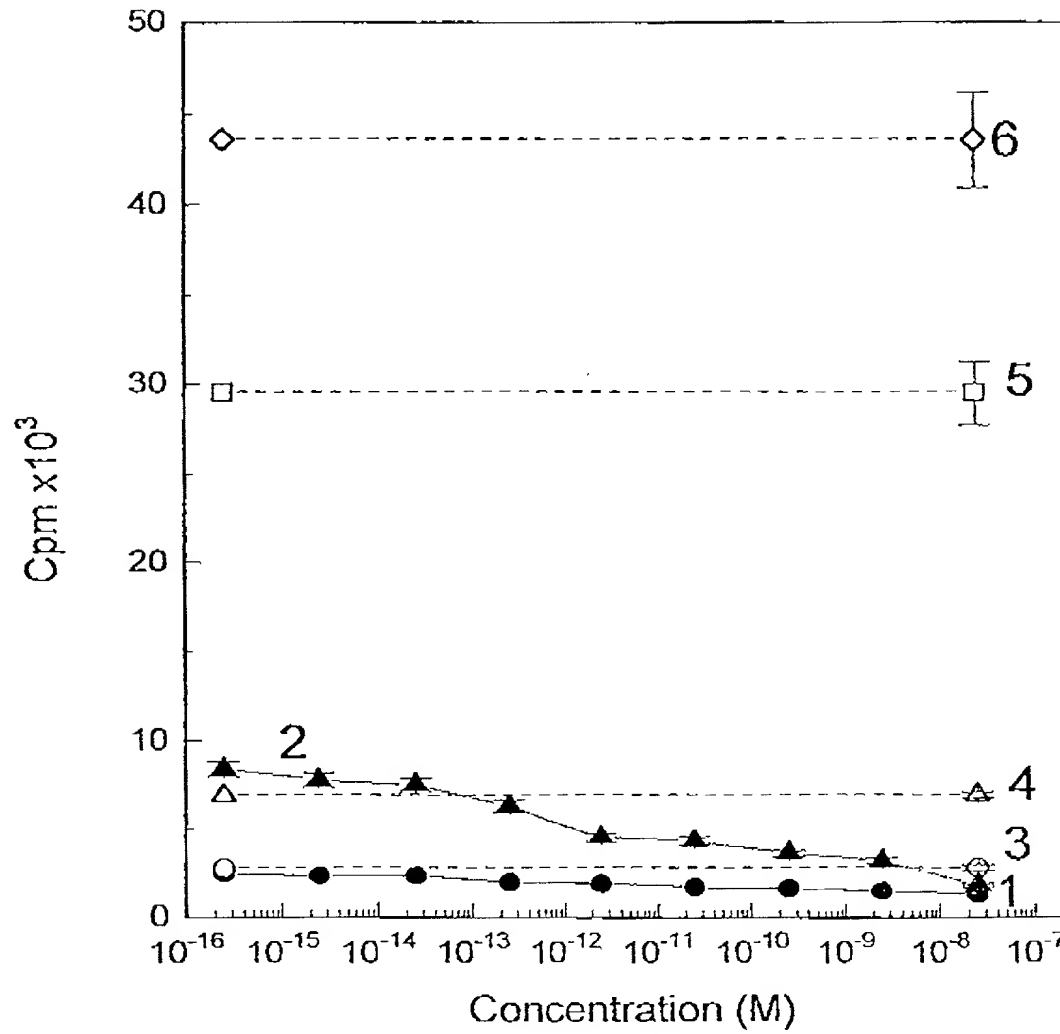


Fig. 5

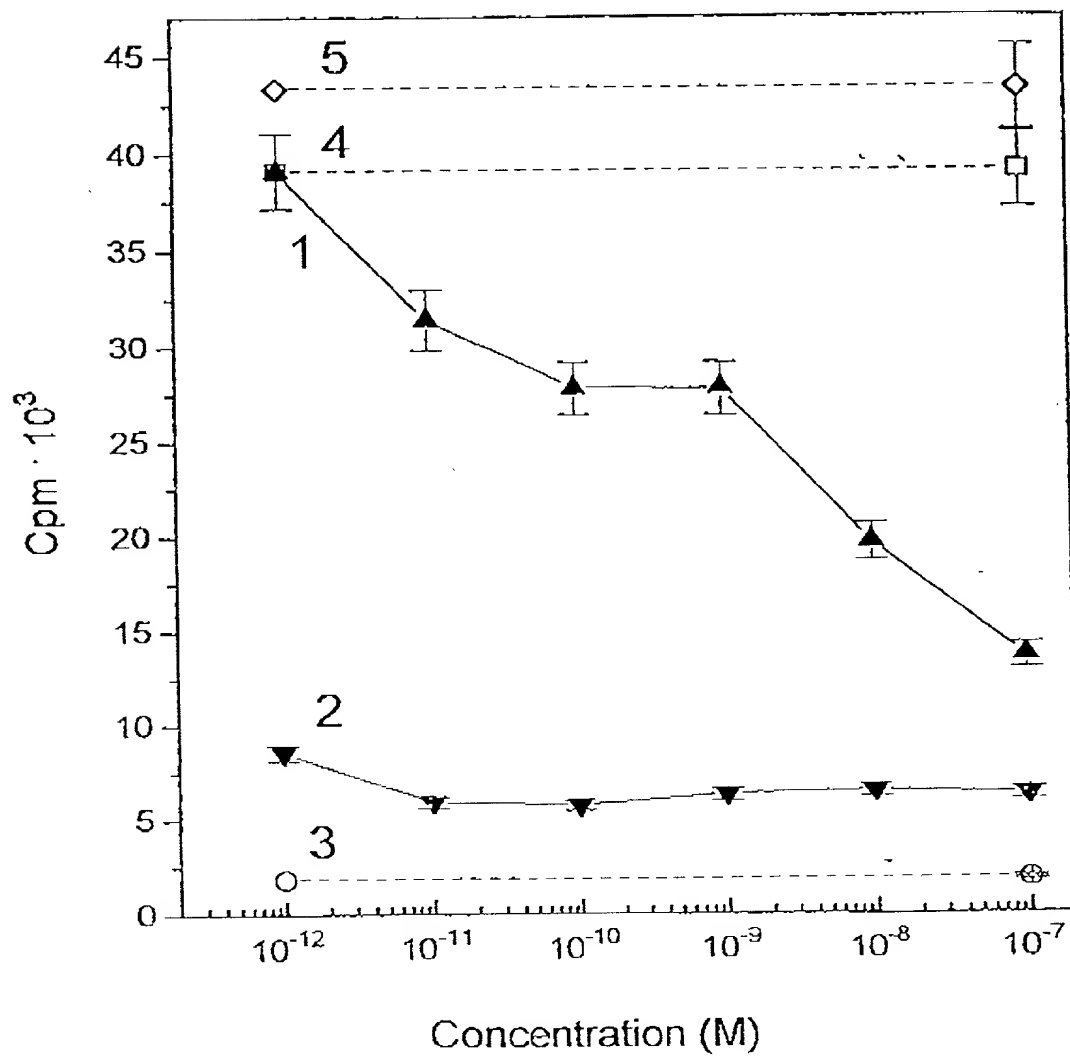


Fig. 6

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COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT AND DESIGN APPLICATIONS

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Insert Title:

"Compositions for Enhancing Immunosuppressants' Pharmaceutical Activities"

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the specification of which is attached hereto. If not attached hereto,

the specification was filed on _____ as
United States Application Number _____;
and amended on _____ (if applicable) and/or
the specification was filed on May 18, 1998 as PCT
International Application Number PCT/FT98/00418;
amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representative or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Insert Priority
Information:
(if appropriate)

Prior Foreign Application(s)

Priority Claimed

<u>972121</u> (Number)	<u>FINLAND</u> (Country)	<u>5/19/1997</u> (Month/Day/Year Filed)	<input checked="" type="checkbox"/> Yes	<input type="checkbox"/> No
_____ (Number)	_____ (Country)	_____ (Month/Day/Year Filed)	<input type="checkbox"/> Yes	<input type="checkbox"/> No
_____ (Number)	_____ (Country)	_____ (Month/Day/Year Filed)	<input type="checkbox"/> Yes	<input type="checkbox"/> No
_____ (Number)	_____ (Country)	_____ (Month/Day/Year Filed)	<input type="checkbox"/> Yes	<input type="checkbox"/> No

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below.

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_____ (Application Number)	_____ (Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More than 12 Months (6 Months for Designs) Prior to the Filing Date of This Application:

Country	Application Number	Date of Filing (Month/Day/Year)
_____	_____	_____
_____	_____	_____

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I hereby claim the benefit under Title 35, United States Code, §120 of any United States and/or PCT application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States and/or PCT application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Insert Prior U.S.
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_____ (Application Number)	_____ (Filing Date)	_____ (Status - patented, pending, abandoned)
_____ (Application Number)	_____ (Filing Date)	_____ (Status - patented, pending, abandoned)

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I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

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